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Detergent-Resistant Membrane Microdomains in the Disposition of the Lipid Signaling Molecule Anandamide

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ABSTRACT

The endogenous cannabinoid anandamide is an agonist of the cannabinoid receptors CB1 and CB2, as well as transient receptor potential vanilloid type 1 and type 4 ion channels. In recent years, there has been a great deal of interest in the cellular processes regulating the signaling of endocannabinoids such as anandamide. This is due largely to evidence that augmentation of cannabinergic tone might be therapeutically beneficial in the treatment of multiple disease states such as chronic pain, anxiety, multiple sclerosis, and neuropsychiatric disorders. Of particular interest are the cellular processes that regulate the cellular accumulation and metabolism of anandamide. Characterization of the process by which anandamide is internalized and metabolized by the cell may identify drug targets useful in the positive modulation of cannabinergic tone. Recently, we reported that detergent-resistant membrane microdomains known as lipid rafts play a role in the cellular accumulation of anandamide by mediating an endocytic process responsible for anandamide internalization. The enzyme primarily responsible for anandamide metabolism, fatty acid amide hydrolase, is excluded from lipid rafts. However, the metabolites of anandamide accumulate in these detergent-resistant membrane microdomains. There is some preliminary evidence that makes it reasonable to propose that anandamide metabolites enriched in lipid rafts may act as precursors to anandamide synthesis. Overall, experimental evidence is mounting that detergent-resistant membrane microdomains such as lipid rafts may play a role in the cellular regulation of anandamide inactivation and production.

KEYWORDS: Anandamide, endocannabinoid, lipid raft, NAPE-PLD, marijuana

INTRODUCTION

Anandamide (AEA) is an endogenously occurring compound that has agonist activity at the cannabinoid 1 (CB1) and cannabinoid 2 (CB2) receptors. CB1 and CB2 receptors are the pharmacological target of plant-derived cannabinoids, such as Δ^9-tetrahydrocannabinol, that may provide therapeutic benefits in the treatment of conditions such as chronic pain, anorexia, autoimmune disease, and anxiety.[1-3] Since the discovery of AEA in 1992,[4] other endocannabinoids, including 2-arachidonylglycerol (2-AG),[5] noladin ether,[6] and virodhamine,[7] have also been identified. While each of the identified endocannabinoid compounds has been the focus of studies aimed at elucidating the mechanisms of endocannabinoid-mediated signaling, AEA and 2-AG are by far the most extensively studied, and their pharmacologic profiles are very similar. Not only are AEA and 2-AG both agonists of the CB1 and CB2 receptors, they also display agonist activity at the vanilloid-type transient receptor potential (TRPV) cation channels TRPV1 and TRPV4.[8,9] Both AEA and 2-AG are synthesized and released in a stimulus-dependent fashion from membrane precursors, undergo cellular uptake by multiple cell types, and are metabolized intracellularly. Fatty acid amide hydrolase (FAAH) is the major enzyme responsible for AEA metabolism, which produces arachidonic acid and ethanolamine,[10] while 2-AG is primarily metabolized by the enzyme monoacylglycerol lipase to form arachidonic acid and glycerol in vivo.[11,12] Although some differences in the cellular processes regulating AEA and 2-AG uptake, metabolism, and synthesis have been reported, the focus of this mini-review will be on cellular mechanisms that may be important to the regulation of AEA signaling. Over the last decade there has been growing interest in the mechanisms that govern the cellular uptake, processing, and synthesis of AEA. This is due largely to the fact that experimental evidence has suggested that in vivo augmentation of AEA levels, and thus, cannabinergic tone, may be therapeutically beneficial in the treatment of such medical conditions as neuropsychiatric disorders,[13,14] chronic pain,[15-17] anxiety,[18] spasticity,[16] and cancer/cellular proliferation.[19] The ability to enhance cannabinergic tone will be dependent on discoveries leading to a better understanding of how cells process and produce AEA as well as other endocannabinoids.

The highly disordered phospholipid bilayer that constitutes the plasma membrane contains specialized membrane microdomains that are enriched in cholesterol, sphingolipids,

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plasmenylethanolamine, and arachidonic acid.[20,21] The liquid-ordered state of these domains imparts a resistance to solubilization with non-ionic detergents.[22] Collectively, these detergent-resistant membrane microdomains have been referred to as lipid rafts. Caveolae are a subclass of lipid rafts that occur as flask-shaped invaginations in the plasma membrane and contain a family of integral membrane proteins called the caveolins.[21,23] Detergent-resistant membrane microdomains, or lipid rafts, are important in the regulation of multiple cellular processes. Recent evidence suggests that lipid rafts/caveolae play a role in the cellular processing and regulation of AEA.[24-27] While studies have implicated lipid rafts in the cellular regulation of AEA, it is possible that other endocannabinoids such as 2-AG are subject to similar regulatory processes. This mini-review will focus on recent studies indicating an important role for detergent-resistant membrane microdomains/lipid rafts in the cellular mechanisms that regulate AEA uptake, metabolism, and biosynthesis.

Detergent-Resistant Membrane Microdomains Play a Role in AEA Uptake

AEA transport has been characterized as rapid, temperature dependent, inhibited by select fatty acid amides, saturable, and independent of ion gradients or adenosine triphosphate hydrolysis.[28-32] Several theories have been proposed in regard to the mechanisms governing AEA transport. It has been suggested that AEA, because of its lipophilic nature, crosses the plasma membrane by simple diffusion[33] and that FAAH is the driving force in this scenario, perpetuating an inward concentration gradient of AEA. AEA uptake also has been proposed to occur by facilitated diffusion via a membrane carrier protein.[19,34,35] This model also recognizes FAAH as a driving force in the cellular accumulation of AEA because FAAH activity would help to maintain an inward concentration gradient of AEA. Other observations have indicated that AEA is sequestered in a yet-to-be-identified cellular compartment that allows the accumulation of AEA to occur well beyond its concentration gradient.[36] Recently, the CB1 receptor has been implicated in the cellular uptake of AEA.[37] Given the diversity and the large number of cell types that have been shown to accumulate AEA, it is not surprising that many different hypotheses regarding the mechanism of AEA transport have been proposed. Here we examine the role that lipid rafts might play in the cellular uptake of AEA.

Experimental evidence has suggested that AEA transport is to some extent regulated by the detergent-resistant lipid raft membrane microdomains. Pharmacological disruption of lipid rafts by cholesterol depletion that inhibits caveolae-related (clathrin-independent) endocytosis reduced AEA uptake in RBL-2H3 cells by ~50%.[38] Furthermore, fluorescence derived from the fluorescent AEA analog SKM 4-45-1[39] colocalized with markers for lipid rafts at early time points following treatment of RBL-2H3 cells with SKM 4-45-1.[38] A model in which AEA is internalized by a caveolae-related endocytic process fits well with the accepted profile of AEA transport. This model also makes allowance for an FAAH-mediated component of AEA transport in that FAAH could indeed augment the internalization of AEA by efficiently clearing intact AEA from endocytic machinery. Recent evidence further supports the hypothesis that AEA internalization might occur by a caveolae-related endocytic process. Bari et al showed that depletion of cholesterol, and thus detergent-resistant membrane domains/lipid rafts, by methyl-β-cyclodextrin reduced AEA internalization by rat C6 glioma cells by ~50%.[27] Data from this study also revealed a B_{max} value for CB1 receptors that was doubled following treatment with methyl-β-cyclodextrin.[27] Conversely, rat C6 glioma cells treated with cholesterol exhibited a decrease in B_{max} value for the CB1 receptor as determined by [^{3}H]CP-55,940 radioligand binding while displaying AEA uptake activity that was ~180% greater than that observed in cells that were not enriched with exogenous cholesterol.[40] Interestingly, the effect of cholesterol on CB1 receptors did not include an increase in actual CB1 receptor protein in the membrane.[40] As discussed by Bari et al, both cholesterol depletion and cholesterol enrichment will alter the membrane fluidity, and thus, the internalization of a putative AEA transport/binding protein resident in the detergent-resistant lipid rafts.[40] Cholesterol enrichment should decrease the membrane fluidity and thus cause an increased level of AEA transport/binding protein available on the cell surface.[40] Such a scenario would cause an increase in the V_{max} of the AEA uptake process, as was reported by the authors.[40] However, cholesterol depletion in rat C6 glioma cells causes an increase in membrane fluidity and consequently an increase in the internalization of the AEA transport/binding protein, resulting in a decreased V_{max} of the AEA uptake process.[27] In contrast to the above researchers, Sandberg and Fowler found that cholesterol depletion has no effect on AEA uptake in P19 cells.[41] This could be due to the fact that detergent-resistant membrane domain-mediated endocytosis, like many other factors, probably has a different role in the cellular accumulation of AEA depending on the cell type.

AEA Metabolites Accumulate in Detergent-Resistant Membrane Microdomains

FAAH is the enzyme that is primarily responsible for the metabolism of AEA.[10] Immunocytochemical studies have indicated that FAAH is localized to intracellular membrane compartments.[42,43] Further characterization of FAAH

localization in Cos-7 cells transfected with cDNA for rat FAAH suggests that FAAH appears to be perinuclear, possibly residing in or near the endoplasmic reticulum.[44] Immunocytochemical studies from our laboratory have confirmed that FAAH is located intracellularly and is potentially colocalized with cellubrevin, a protein involved the function of endocytic recycling pathways (Figure 1).

Our laboratory has shown that RBL-2H3 cells treated with [^{3}H]AEA for 5 minutes and then fractionated by sucrose gradient centrifugation reveal an enrichment of tritium in the detergent-resistant membrane microdomains/lipid rafts.[25] This result was observed when AEA was radiolabeled on both the arachidonate backbone and the ethanolamine portion of the molecule. The fractionation technique used in this experiment was not capable of distinguishing between subsets of lipid rafts. However, Western blot analysis revealed that the isolated fractions were rich in the lipid raft markers caveolin-1 and flotilin-1 and were thus referred to as caveolin-rich membrane fractions. Parallel experiments were also conducted by treating RBL-2H3 cells with unlabeled AEA for 5 minutes and then isolating the caveolin-rich membrane fractions. Liquid chromatography, tandem mass spectrometry (LC/MS/MS) analysis of these fractions revealed very low levels of intact AEA, indicating that the observed enrichment of tritium in caveolin-rich membrane fractions was representative of AEA metabolites and not intact AEA.[25] These results suggest that following uptake, AEA is trafficked to intracellular compartments containing FAAH, where it is metabolized, and that these metabolites are then trafficked back to the plasma membrane, where they accumulate in detergent-resistant membrane fractions. HeLa cells do not express detectable levels of FAAH, but they do accumulate AEA.[45] HeLa cells that were treated with [^{3}H]AEA for 5 minutes displayed no significant accumulation of tritium in their caveolin-rich membrane fractions.[25] However, an accumulation of tritium in the caveolin-rich membrane fraction was observed if HeLa cells were transfected with FAAH cDNA prior to treatment with [^{3}H]AEA. The fact that AEA-derived tritium was observed in the detergent-resistant membrane fraction only when FAAH was present in the HeLa cells further supports the hypothesis that following metabolism by FAAH, AEA's metabolites, probably reesterified into phospholipids, are rapidly trafficked back to detergent-resistant membrane domains.

Are Detergent-Resistant Membrane Microdomains Involved in AEA Synthesis?

Like other endocannabinoids, AEA is synthesized and released "on demand." Two steps govern the overall formation of AEA. First, the enzyme N-acyl-transferase promotes the transfer of a fatty acyl chain (arachidonate) from the sn-1 position of a phospholipid to the primary amine of phosphatidylethanolamine to form N-arachidonylphosphatidylethanolamine (NAPE).[29,46] Second, NAPE is cleaved by a calcium-dependent phospholipase D (PLD) that is specific for N-acylethanolamides (NAPE-PLD) to form AEA.[29,47] While these 2 enzymatic processes are well understood, the exact cellular organization and localization of the components involved in the synthesis of AEA are yet to be fully characterized.

The presence of AEA metabolites in a well-ordered membrane compartment where they could serve as precursors for AEA synthesis would offer obvious advantages for efficient biosynthesis. Note that PLD activity has been reported to be enriched in caveolin-rich membrane microdomains.[48] Do detergent-resistant membrane microdomains accumulate AEA metabolites as precursors to form new AEA? Do they serve as the location for AEA synthesis? Recently, our laboratory conducted preliminary studies in which RBL-2H3 cells were treated with [^{3}H]AEA for 24 hours to allow

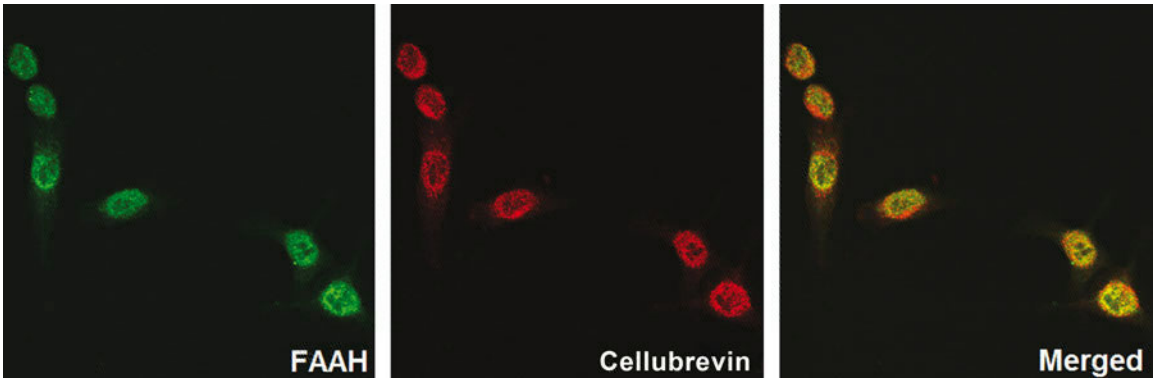

Figure 1. Immunocytochemical localization of FAAH and cellubrevin. Confocal microscopy analysis shows that FAAH is colocalized with cellubrevin in RBL-2H3 cells. (Left) FAAH was labeled with rabbit anti-FAAH (1:3000) followed by donkey antirabbit immunoglobulin G conjugated to (green) Alexa Fluor 488 (Molecular Probes) (1:3000). (Middle) Cellubrevin was labeled with mouse anticellubrevin (1:1000) followed by goat antimouse IgG (1:2000) conjugated to (red) Alexa Fluor 568 (Molecular Probes). (Right) Overlay of FAAH and cellubrevin showing colocalization of the 2 proteins. FAAH indicates fatty acid amide hydrolase.

for complete metabolism and for the incorporation of [^{3}H]AEA metabolites into the cellular pool as potential precursors of new AEA. Following the 24-hour incubation, cells were washed and treated for 10 minutes with either 1 μM ionomycin or vehicle in the presence of 5 μM methylarachidonyl fluorophosphonate to prevent AEA metabolism. Ionomycin is a calcium ionophore activating the calcium-dependent NAPE-PLD and initiating AEA synthesis. After the ionomycin incubation, the reaction buffer was collected for lipid extraction and analysis by thin-layer chromatography (TLC). TLC analysis revealed a robust increase in the levels of AEA, 2-AG, and arachidonic acid present in the reaction buffer following ionomycin treatment when compared with control (Figure 2). These results offer the first evidence that following metabolism and reincorporation into the membrane, AEA can be recycled into new endocannabinoid molecules. Interestingly, recent reports have shown that neither cholesterol depletion nor enrichment in rat C6 glioma affect NAPE-PLD activity,[27,40] although the reports did not measure actual AEA production and release. These latter findings could suggest that detergent-resistant membrane domains do not play a crucial role in AEA synthesis. The method used in this study is appropriate for assessing the overall NAPE-PLD enzyme activity in a cell. However, it is important to note that the above NAPE-PLD activity assays were performed on cell homogenates of unstimulated cells. Therefore, the impact of cholesterol depletion/enrichment on the availability of endogenously occurring NAPE and NAPE-PLD activity in whole cells remains unknown. One plausible scenario is that NAPE-PLD is recruited to detergent-resistant domains only after a cellular stimulus to increase the synthesis of AEA, that is, an increase in intracellular calcium. Likewise, detergent-resistant membrane domains may serve as only a reservoir for AEA metabolites/precursors, with the actual cleavage of NAPE occurring elsewhere in the membrane. Additional data are needed to further define the role of detergent-resistant membrane domains in AEA synthesis. However, preliminary evidence suggests that further investigation into the cellular localization of NAPE-PLD as well as the role that detergent-resistant membrane domains play in the synthesis of AEA and other endocannabinoids is merited.

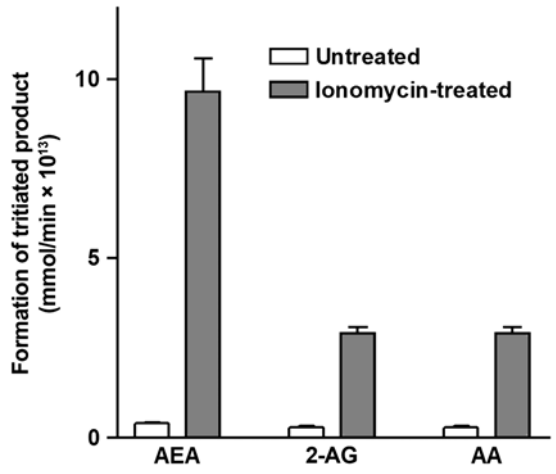

Figure 2. Release of [^{3}H] AEA-derived AEA, 2-AG, and AA from RBL-2H3 cells. RBL-2H3 cells were prelabeled with 1 nM [^{3}H] AEA for 24 hours. Cells were then treated with methylarachidonyl fluorophosphonate (5 μM, 10 minutes, 37°C) followed by a treatment with ionomycin (1 μM, 10 minutes, 37°C) or buffer control to evoke synthesis of [^{3}H] products. Reaction was terminated by the addition of ice-cold 2:1 chloroform:methanol mixture (vol/vol), and the organic layer was extracted. Reextraction was performed by the addition of 1 mL of chloroform. Lipids were then separated by thin-layer chromatography with 13:5 isooctane:ethyl acetate saturated with water. Product spots were visualized by iodine vapor. The distribution of radioactivity in the lipid spots was quantified using a Beckman scintillation counter. AEA indicates anandamide; 2-AG, 2-arachidonyl glycerol; AA, arachidonic acid.

Conclusion

Detergent-resistant membrane microdomains, or lipid rafts, seem to play an important role in the overall regulation of AEA and perhaps other endocannabinoids. The extent to which the reported dependence of AEA uptake on intact detergent-resistant membrane domains applies to other cell lines is not yet clear. However, there is clear evidence that a caveolae-related endocytic process is involved in the cellular accumulation of AEA in some cell lines and that in these cells the metabolites of AEA are trafficked back to the plasma membrane, where they accumulate in these detergent-resistant membrane domains as well. We also hypothesize that detergent-resistant membrane domains are the site of AEA synthesis. Data from our preliminary studies along with the observation that the biosynthesis of another hydrophobic amide signaling molecule, ceramide, is localized to caveolae[49] make detergent-resistant membrane domains a worthy candidate as the location of AEA synthesis. There is still much to learn about the relationship between detergent-resistant membrane microdomains and endocannabinoid-mediated signaling. For now, we must continue to explore the role that detergent-resistant membrane microdomains and the cellular processes that they mediate play in AEA internalization and processing, as well as the role that they may play in AEA synthesis. A better understanding of these processes will hopefully provide insight into new strategies for the pharmacotherapeutic modulation of the endocannabinoid signaling system.

References

1. Howlett AC. Pharmacology of cannabinoid receptors. *Annu Rev Pharmacol Toxicol*. 1995;35:607-634.

2. Di Marzo V. 'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties: biochemistry and possible physiopathological relevance. *Biochim Biophys Acta*. 1998;1392:153-175.

3. Porter AC, Felder CC. The endocannabinoid nervous system: unique opportunities for therapeutic intervention. *Pharmacol Ther*. 2001;90:45-60.

4. Devane WA, Hanus L, Breuer A, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*. 1992;258:1946-1949.

5. Sugiura T, Kondo S, Sukagawa A, et al. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun*. 1995;215:89-97.

6. Hanus L, Abu-Lafi S, Fride E, et al. 2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. *Proc Natl Acad Sci USA*. 2001;98:3662-3665.

7. Porter AC, Sauer JM, Knierman MD, et al. Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor. *J Pharmacol Exp Ther*. 2002;301:1020-1024.

8. McVey DC, Schmid PC, Schmid HH, Vigna SR. Endocannabinoids induce ileitis in rats via the capsaicin receptor (VR1). *J Pharmacol Exp Ther*. 2003;304:713-722.

9. Nilius B, Vriens J, Prenen J, Droogmans G, Voets T. TRPV4 calcium entry channel: a paradigm for gating diversity. *Am J Physiol Cell Physiol*. 2004;286:C195-C205.

10. Ueda N, Yamamoto S. Anandamide amidohydrolase (fatty acid amide hydrolase). *Prostaglandins Other Lipid Mediat*. 2000;61:19-28.

11. Dinh TP, Carpenter D, Leslie FM, et al. Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc Natl Acad Sci USA*. 2002;99:10819-10824.

12. Dinh TP, Freund TF, Piomelli D. A role for monoglyceride lipase in 2-arachidonoylglycerol inactivation. *Chem Phys Lipids*. 2002;121:149-158.

13. Jarrahian A, Watts VJ, Barker EL. D2 dopamine receptors modulate Galpha-subunit coupling of the CB1 cannabinoid receptor. *J Pharmacol Exp Ther*. 2004;308:880-886.

14. Beltramo M, de Fonseca FR, Navarro M, et al. Reversal of dopamine D(2) receptor responses by an anandamide transport inhibitor. *J Neurosci*. 2000;20:3401-3407.

15. Cravatt BF, Demarest K, Patricelli MP, et al. Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci USA*. 2001;98:9371-9376.

16. de Lago E, Ligresti A, Ortar G, et al. In vivo pharmacological actions of two novel inhibitors of anandamide cellular uptake. *Eur J Pharmacol*. 2004;484:249-257.

17. Lopez-Rodriguez ML, Viso A, Ortega-Gutierrez S, et al. Design, synthesis and biological evaluation of new endocannabinoid transporter inhibitors. *Eur J Med Chem*. 2003;38:403-412.

18. Kathuria S, Gaetani S, Fegley D, et al. Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med*. 2003;9:76-81.

19. Ruiz-Llorente L, Ortega-Gutierrez S, Viso A, et al. Characterization of an anandamide degradation system in prostate epithelial PC-3 cells: synthesis of new transporter inhibitors as tools for this study. *Br J Pharmacol*. 2004;141:457-467.

20. Brown DA, London E. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J Biol Chem*. 2000;275:17221-17224.

21. Pike LJ, Han X, Chung KN, Gross RW. Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. *Biochemistry*. 2002;41:2075-2088.

22. London E, Brown DA. Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). *Biochim Biophys Acta*. 2000;1508:182-195.

23. Razani B, Woodman SE, Lisanti MP. Caveolae: from cell biology to animal physiology. *Pharmacol Rev*. 2002;54:431-467.

24. McFarland MJ, Barker EL. Lipid rafts: a nexus for endocannabinoid signaling? *Life Sci*. 2005;77:1640-1650.

25. McFarland MJ, Porter AC, Rakhshan FR, Rawat DS, Gibbs RA, Barker EL. A role for caveolae/lipid rafts in the uptake and recycling of the endogenous cannabinoid anandamide. *J Biol Chem*. 2004;279:41991-41997.

26. Sarker KP, Maruyama I. Anandamide induces cell death independently of cannabinoid receptors or vanilloid receptor 1: possible involvement of lipid rafts. *Cell Mol Life Sci*. 2003;60:1200-1208.

27. Bari M, Battista N, Fezza F, Finazzi-Agro A, Maccarrone M. Lipid rafts control signaling of type-1 cannabinoid receptors in neuronal cells: implications for anandamide-induced apoptosis. *J Biol Chem*. 2005;280:12212-12220.

28. Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A, Piomelli D. Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science*. 1997;277:1094-1097.

29. Di Marzo V, Fontana A, Cadas H, et al. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature*. 1994;372:686-691.

30. Hillard CJ, Edgemond WS, Jarrahian A, Campbell WB. Accumulation of N-arachidonoylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion. *J Neurochem*. 1997;69:631-638.

31. Maccarrone M, van der Stelt M, Rossi A, Veldink GA, Vliegenthart JF, Agro AF. Anandamide hydrolysis by human cells in culture and brain. *J Biol Chem*. 1998;273:32332-32339.

32. Rakhshan F, Day TA, Blakely RD, Barker EL. Carrier-mediated uptake of the endogenous cannabinoid anandamide in RBL-2H3 cells. *J Pharmacol Exp Ther*. 2000;292:960-967.

33. Glaser ST, Abumrad NA, Fatade F, Kaczocha M, Studholme KM, Deutsch DG. Evidence against the presence of an anandamide transporter. *Proc Natl Acad Sci USA*. 2003;100:4269-4274.

34. Ligresti A, Morera E, Van Der Stelt M, et al. Further evidence for the existence of a specific process for the membrane transport of anandamide. *Biochem J*. 2004;380:265-272.

35. Ortar G, Ligresti A, De Petrocellis L, Morera E, Di Marzo V. Novel selective and metabolically stable inhibitors of anandamide cellular uptake. *Biochem Pharmacol*. 2003;65:1473-1481.

36. Hillard CJ, Jarrahian A. Cellular accumulation of anandamide: consensus and controversy. *Br J Pharmacol*. 2003;140:802-808.

37. Ortega-Gutierrez S, Hawkins EG, Viso A, Lopez-Rodriguez ML, Cravatt BF. Comparison of anandamide transport in FAAH wild-type and knockout neurons: evidence for contributions by both FAAH and the CB1 receptor to anandamide uptake. *Biochemistry*. 2004;43:8184-8190.

38. McFarland MJ, Porter AC, Rakhshan FR, Rawat DS, Gibbs RA, Barker EL. A role for caveolae/lipid rafts in the uptake and recycling of the endogenous cannabinoid anandamide. *J Biol Chem*. 2004;279:41991-41997.

39. Muthian S, Nithipatikom K, Campbell WB, Hillard CJ. Synthesis and characterization of a fluorescent substrate for the N-arachidonoylethanolamine (anandamide) transmembrane carrier. *J Pharmacol Exp Ther*. 2000;293:289-295.

40. Bari M, Paradisi A, Pasquariello N, Maccarrone M. Cholesterol-dependent modulation of type 1 cannabinoid receptors in nerve cells. *J Neurosci Res*. 2005;81:275-283.

41. Sandberg A, Fowler CJ. Measurement of saturable and non-saturable components of anandamide uptake into P19 embryonic carcinoma cells in the presence of fatty acid-free bovine serum albumin. *Chem Phys Lipids*. 2005;134:131-139.

42. Egertova M, Giang DK, Cravatt BF, Elphick MR. A new perspective on cannabinoid signalling: complementary localization of fatty acid amide hydrolase and the CB1 receptor in rat brain. *Proc Biol Sci.* 1998;265:2081-2085.

43. Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature*. 1996;384:83-87.

44. Giang DK, Cravatt BF. Molecular characterization of human and mouse fatty acid amide hydrolases. *Proc Natl Acad Sci USA*. 1997;94:2238-2242.

45. Day TA, Rakhshan F, Deutsch DG, Barker EL. Role of fatty acid amide hydrolase in the transport of the endogenous cannabinoid anandamide. *Mol Pharmacol*. 2001;59:1369-1375.

46. Cadas H, di Tomaso E, Piomelli D. Occurrence and biosynthesis of endogenous cannabinoid precursor, N-arachidonoyl phosphatidylethanolamine, in rat brain. *J Neurosci*. 1997;17: 1226-1242.

47. Okamoto Y, Morishita J, Tsuboi K, Tonai T, Ueda N. Molecular characterization of a phospholipase D generating anandamide and its congeners. *J Biol Chem*. 2004;279:5298-5305.

48. Czarny M, Lavie Y, Fiucci G, Liscovitch M. Localization of phospholipase D in detergent-insoluble, caveolin-rich membrane domains: modulation by caveolin-1 expression and caveolin-182-101. *J Biol Chem*. 1999;274:2717-2724.

49. Dobrowsky RT. Sphingolipid signalling domains floating on rafts or buried in caves? *Cell Signal*. 2000;12:81-90.